APPENDIX A

TABLE 3

Crystal Formation by the Hybrid δ-Endotoxins

Strain	Plasmid	Parent δ-Endotoxins	Crystal Formation
EG11060	pEG1065	Cry1Ac + Cry1F	+
EG11062	pEG1067	Cry1Ac + Cry1F	+
EG11063	pEG1068	Cry1Ac + Cry1F	+
EG11065	pEG1070	Cry1Ac + Cry1F	−
EG11067	pEG1072	Cry1Ac + Cry1F	−
EG11071	pEG1074	Cry1Ac + Cry1F	+
EG11073	pEG1076	Cry1Ac + Cry1F	+
EG11074	pEG1077	Cry1Ac + Cry1F	+
EG11087	pEG1088	Cry1Ac + Cry1C	−
EG11088	pEG1089	Cry1F + Cry1Ac	−
EG11090	pEG1091	Cry1C + Cry1Ac	−
EG11091	pEG1092	Cry1Ac + Cry1C	+
EG11092	pEG1093	Cry1Ab + Cry1Ac + Cry1F	+
EG11735	pEG365	Cry1Ab + Cry1F + Cry1Ac	+
EG11751	pEG378	Cry1Ac + Cry1F	+

The δ-endotoxin production for some of the *B. thuringiensis* strains specified in TABLE 3 was examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Baum *et al.*, 1990. Equal volume cultures of each *B. thuringiensis* strain were grown in C-2 medium until fully sporulated and lysed. The cultures were centrifuged and the spore/crystal pellet was washed twice with equal volumes of distilled deionized water. The final pellet was suspended in half the culture volume of 0.005% Triton X-100®. An equal volume of each washed culture was analyzed by SDS-PAGE as shown in FIG. 2.

The majority of hybrids involving Cry1Ac and Cry1F formed stable crystals in *B. thuringiensis* A notable exception is EG11088 in which the active toxin fragment would

be the reciprocal exchange of EG11063. Two of the three hybrids involving Cry1Ac and Cry1C, EG11087 and EG11090, failed to produce crystal in *B. thuringiensis* even though these reciprocal hybrids mimic the activated toxin fragments of crystal-forming EG11063 and EG11074.

Every strain that was examined by SDS-PAGE produced some level of δ-endotoxin. As expected, however, those cultures identified as crystal negative produced very little protein (*e.g.*, lane e: EG11065, lane f: EG11067, lane j: EG11088, and lane k: EG11090). For reference, typical yields from a crystal forming δ-endotoxin is shown for Cry1Ac (lane a). Several hybrid δ-endotoxins produce comparable levels of protein including EG11060 (lane b), EG11062 (lane c), EG11063 (lane d; SEQ ID NO:10), and EG11074 (lane i; SEQ ID NO:12). The data clearly show that efficient hybrid δ-endotoxin production in *B. thuringiensis* is unpredictable and varies depending on the parent δ-endotoxins used to construct the hybrid.

6.3 Example 3 -- Proteolytic Processing of the Hybrid δ-Endotoxins

Proteolytic degradation of the protoxin form of the δ-endotoxin to a stable active toxin occurs once δ-endotoxin crystals are solubilized in the larval midgut. One measure of the potential activity of δ-endotoxins is the stability of the active δ-endotoxin in a proteolytic environment. To test the proteolytic sensitivity of the hybrid δ-endotoxins, solubilized toxin was subjected to trypsin digestion. The δ-endotoxins were purified from sporulated *B. thuringiensis* cultures and quantified as described by Chambers *et al.*, 1991. Exactly 250 µg of each hybrid δ-endotoxin crystal was solubilized in 30 mM $NaHCO_3$, 10 mM DTT (total volume 0.5 ml). Trypsin was added to the solubilized toxin at a 1:10 ratio. At appropriate time points 50 µl aliquots were removed to 50 µl Laemmli buffer, heated to 100°C for 3 min., and frozen in a dry-ice ethanol bath for subsequent analysis. The trypsin digests of the solubilized toxins were analyzed by SDS-PAGE and the amount of active δ-endotoxin at each time point was quantified by densitometry. A graphic representation of the results from these studies are shown in FIG. 3.

The wild-type Cry1Ac is rapidly processed to the active δ-endotoxin fragment that is stable for the duration of the study. The hybrid δ-endotoxins from EG11063 and EG11074 are also processed to active δ-endotoxin fragments which are stable for the duration of the study. The processing of the EG11063 δ-endotoxin occurs at a slower rate and a higher percentage of this active δ-endotoxin fragment remains at each time point. Although the hybrid δ-endotoxins from EG11060 and EG11062 are process to active δ-endotoxin fragments, these fragments are more susceptible to further cleavage and degrade at various rates during the course of the study. The 5′ exchange points between *Cry1Ac* and *Cry1F* for the EG11062 and EG11063 δ-endotoxins result in toxins that differ by only 21 amino acid residues (see FIG. 1). However, the importance of maintaining *Cry1Ac* sequences at these positions is evident by the more rapid degradation of the EG11062 δ-endotoxin. These data demonstrate that different hybrid δ-endotoxins constructed using the same parental δ-endotoxins can vary significantly in biochemical characteristics such as proteotytic stability.

6.4 Example 4 -- Bioactivity of the Hybrid δ-Endotoxins

B. thuringiensis cultures expressing the desired δ-endotoxin were grown until fully sporulated and lysed and washed as described in Example 2. The δ-endotoxin levels for each culture were quantified by SDS-PAGE as described by Baum *et al.*, 1990. In the case of bioassay screens, a single appropriate concentration of each washed δ-endotoxin culture was topically applied to 32 wells containing 1.0 ml artificial diet per well (surface area of 175 mm^2). A single neonate larvae was placed in each of the treated wells and the tray covered by a clear perforated mylar sheet. Larvae mortality was scored after 7 days of feeding and percent mortality expressed as the ratio of the number of dead larvae to the total number of larvae treated (32).

In the case of LC_{50} determinations (δ-endotoxin concentration giving 50% mortality), δ-endotoxins were purified from the *B. thuringiensis* cultures and quantified as described by Chambers *et al.*, 1991. Eight concentrations of the δ-endotoxins were

prepared by serial dilution in 0.005% Triton X-100® and each concentration was topically applied to wells containing 1.0 ml of artificial diet. Larvae mortality was scored after 7 days of feeding (32 larvae for each δ-endotoxin concentration). In all cases the diluent served as the control.

A comparison of the Cry1A/Cry1F hybrid toxins by bioassay screens is shown in TABLE 4. The hybrid δ-endotoxins from strains EG11063 and EG11074 maintain the activities of the parental Cry1Ac and Cry1F δ-endotoxins. Furthermore, the hybrid δ-endotoxin from EG11735 maintains the activity of its parental Cry1Ab and Cry1F δ-endotoxins. The δ-endotoxins produce by strains EG11061, EG11062, EG11071, and EG11073 have no insecticidal activity on the insect larvae tested despite 1) being comprised of at least one parental δ-endotoxin that is active against the indicated larvae and 2) forming stable, well-defined crystals in *B. thuringiensis*. These results demonstrate the unpredictable nature of hybrid toxin constructions.

For the data in TABLE 4. All strains were tested as washed sporulated cultures. For each insect tested, equivalent amounts of δ-endotoxins were used and insecticidal activity was based on the strain showing the highest percent mortality (++++).

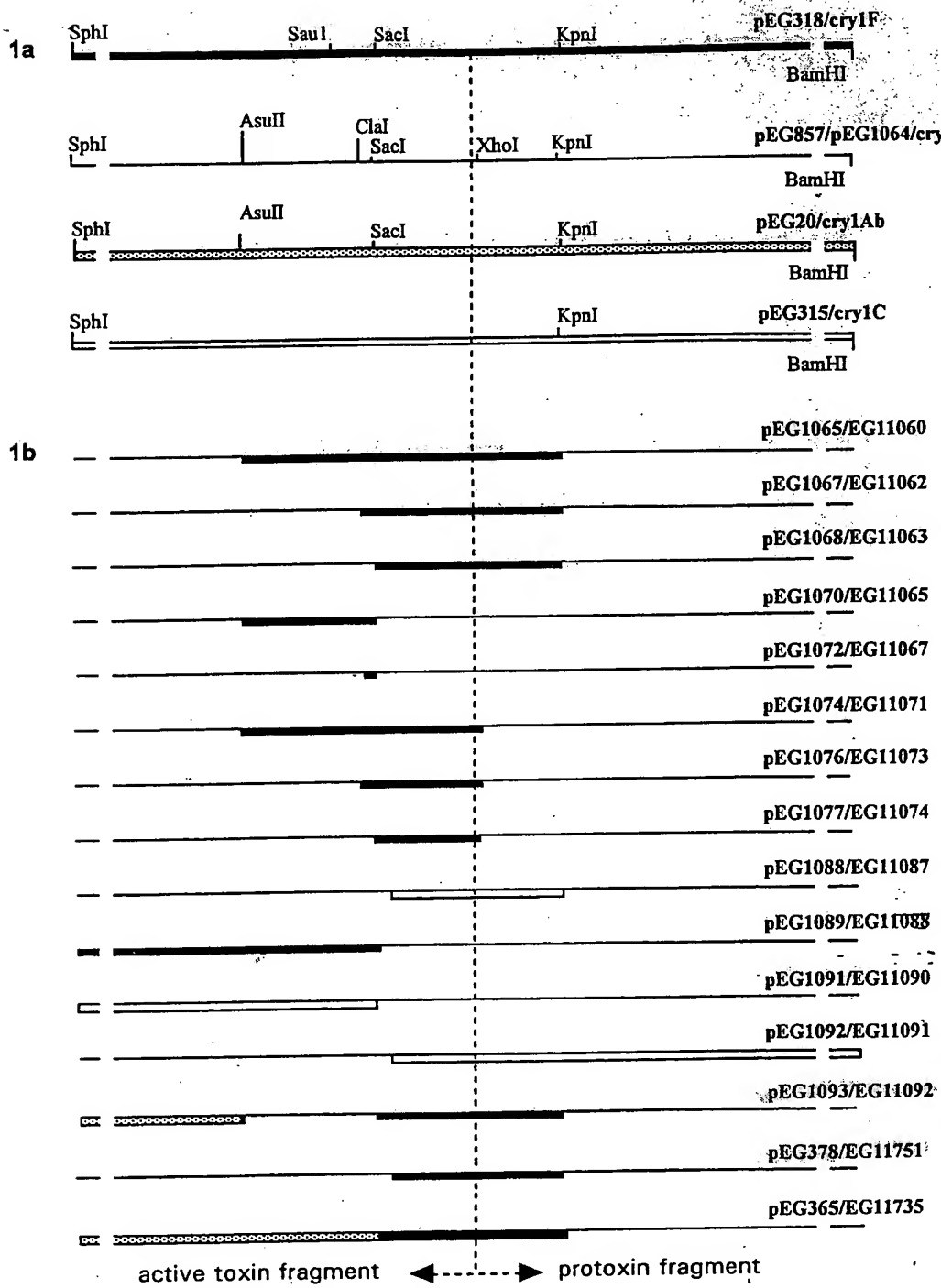
1a
SphI
Sau1
SacI
KpnI
pEG318/cry1F
BamHI
AsuII
ClaI
SacI
XhoI
KpnI
pEG857/pEG1064/cry1Ac
BamHI
AsuII
SacI
KpnI
pEG20/cry1Ab
BamHI
SphI
KpnI
pEG315/cry1C
BamHI
1b
pEG1065/EG11060
pEG1067/EG11062
pEG1068/EG11063
pEG1070/EG11065
pEG1072/EG11067
pEG1074/EG11071
pEG1076/EG11073
pEG1077/EG11074
pEG1088/EG11087
pEG1089/EG11088
pEG1091/EG11090
pEG1092/EG11091
pEG1093/EG11092
pEG378/EG11751
pEG365/EG11735
active toxin fragment
protoxin fragment

Fig. 1

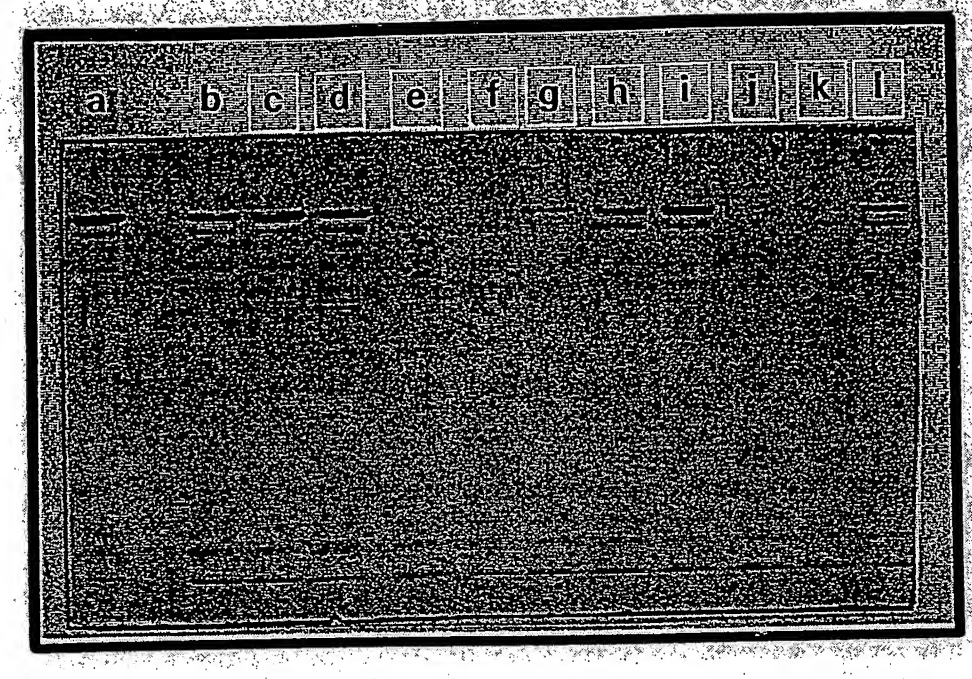

FIG. 2

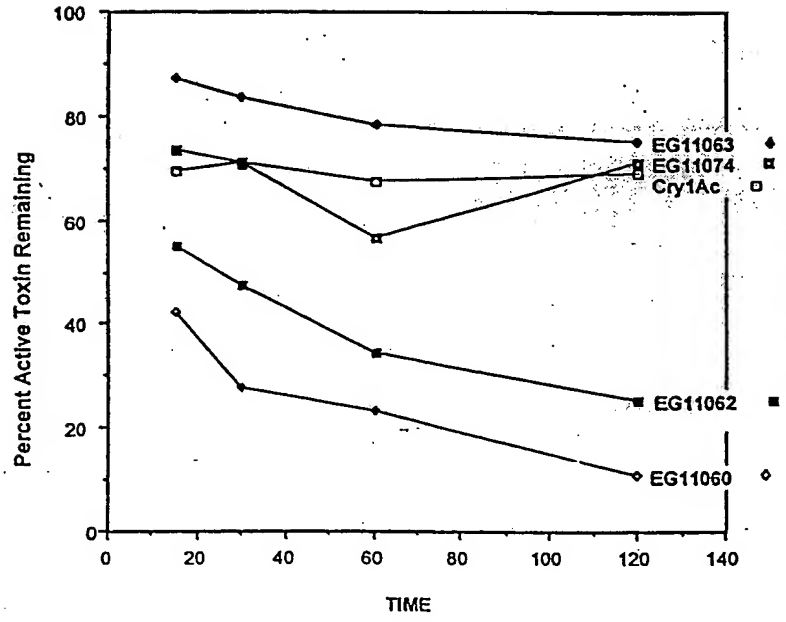
100
80
60
40
20
0
Percent Active Toxin Remaining
0
20
40
60
80
100
120
140
TIME
EG11063
EG11074
Cry1Ac
EG11062
EG11060

FIG. 3